CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9

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Background: Three principal genes are involved in developmental programmed cell death (PCD) in the nematode worm *Caenorhabditis elegans*. The *ced-3* and *ced-4* genes are both required for each PCD, whereas *ced-9* acts to prevent the death-promoting actions of these genes in cells that are destined to survive. Vertebrate homologues of both *ced-3* and *ced-9* have been identified as the genes encoding the caspase cysteine proteases and the Bcl-2 family, respectively. In contrast, no vertebrate homologue of *ced-4* is known. The CED-3/caspases are important effectors of apoptosis that are presumed to act by cleaving specific target substrates. However, the molecular functions of the CED-9/Bcl-2 and CED-4 proteins are unknown. The unicellular yeast *Schizosaccharomyces pombe* shares many general cellular properties with metazoa, but has no identified cell suicide machinery. We have therefore used *S. pombe* as a naive model cell system in which to examine the biological effects of cell-death proteins.

Results: Induction of wild-type *ced-4* expression in *S. pombe* resulted in rapid focal chromatin condensation and lethality. Mutation of the putative nucleotide-binding P-loop motif of CED-4 (K165Q) eliminated the lethal phenotype. Immunolocalization of CED-4 to the condensed chromatin suggested that the phenotype may result from an intrinsic activity of CED-4. Co-expression of *ced-9* prevented CED-4-induced chromatin condensation and lethality, and caused the relocalization of CED-4 to endoplasmic reticulum and outer mitochondrial membranes. A direct interaction between CED-4 and CED-9 was confirmed by yeast two-hybrid analysis.

Conclusions: Using *S. pombe* as a model system in which to assay CED-4 function, we have identified a potential direct role for CED-4 in chromatin condensation. Chromatin condensation is a ubiquitous feature of metazoan apoptosis that has yet to be linked to an effector. The CED-9-mediated rescue of CED-4-induced lethality in this system and the interaction of the two proteins in the yeast two-hybrid analysis suggest that CED-9 inhibits CED-4 action by direct physical association.

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Background

The nematode worm *Caenorhabditis elegans* has proved to be an illuminating model for programmed cell death (PCD) in more complex metazoans. Genetic screens carried out by Horvitz and colleagues [1] identified *ced-9*, *ced-3* and *ced-4* as critical regulators/effectors of apoptosis in *C. elegans*: mutation of either *ced-3* or *ced-4* completely abolishes the developmental PCD that occurs during nematode embryogenesis, whereas *ced-9* acts to restrain *ced-3* and *ced-4* and is required to prevent additional, unscheduled deaths [2]. This basal apoptotic machinery appears to be substantially conserved throughout metazoan evolution [3,4]. Multiple *ced-9* homologues are present in man and include the gene for the *bcl-2* proto-oncogene and its relatives *bcl-x*, *bax*, *bak*, *bcl-w*, *mcl-1* and *A1* [5]. Human *ced-3* homologues comprise the 10 or so known genes encoding mammalian caspases [6] and, more recently, *Drosophila* homologues *drICE* [7] and *DCP-1* [8] have been identified.

The caspases are post-translationally activated by proteolytic cleavage at sites that themselves conform to caspase consensus cleavage sites; the caspases therefore presumably exist in a hierarchical network of autoactivation and transactivation. Neither the key substrates for caspase-induced apoptosis nor the precise interconnections within the caspase cascades have been definitively elucidated, although it has been established that the ultimate action of the caspases is to dismantle the cell by cleavage of specific substrates.

The function of the *ced-9/bcl-2* family is less clearly understood. In vertebrates, both pro- and anti-apoptotic members of the Bcl-2 family have been identified and, even in the nematode, there is evidence that the ostensibly anti-apoptotic archetype, *ced-9*, can promote cell death in certain circumstances [9]. In vertebrates, the pro- and anti-apoptotic members of the Bcl-2 family interact physically to form heterodimers [10], and this may be the method of functional antagonism between certain family members. However, it remains unclear whether the underlying basal action of the Bcl-2 family proteins is the promotion or the inhibition of apoptosis; nor is it known how the activities of the Bcl-2 family proteins are linked to the caspase effectors.

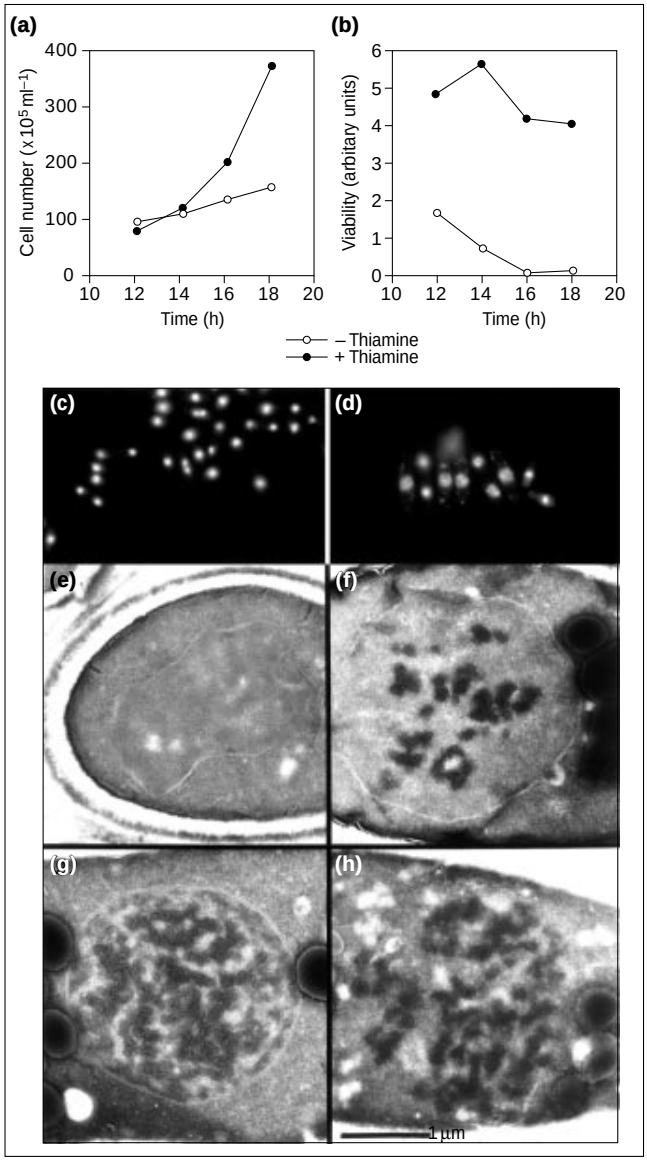
Of the three central players in nematode PCD, only the role of *ced-4* [11] has remained entirely obscure. Recently, the unicellular yeast *S. pombe* has emerged as a possible model cell system in which to study inducers and suppressors of apoptosis. For example, the 'killer' members of the Bcl-2 family, Bax and Bak, induce cell death in *S. pombe* with a phenotype that shares some of the characteristics of metazoan PCD and that is suppressed by the anti-apoptotic Bcl-x_L protein [12]. We therefore undertook to examine the effect of expression of *ced-4* in *S. pombe*.

Results and discussion

Expression of CED-4 protein in *S. pombe* is lethal

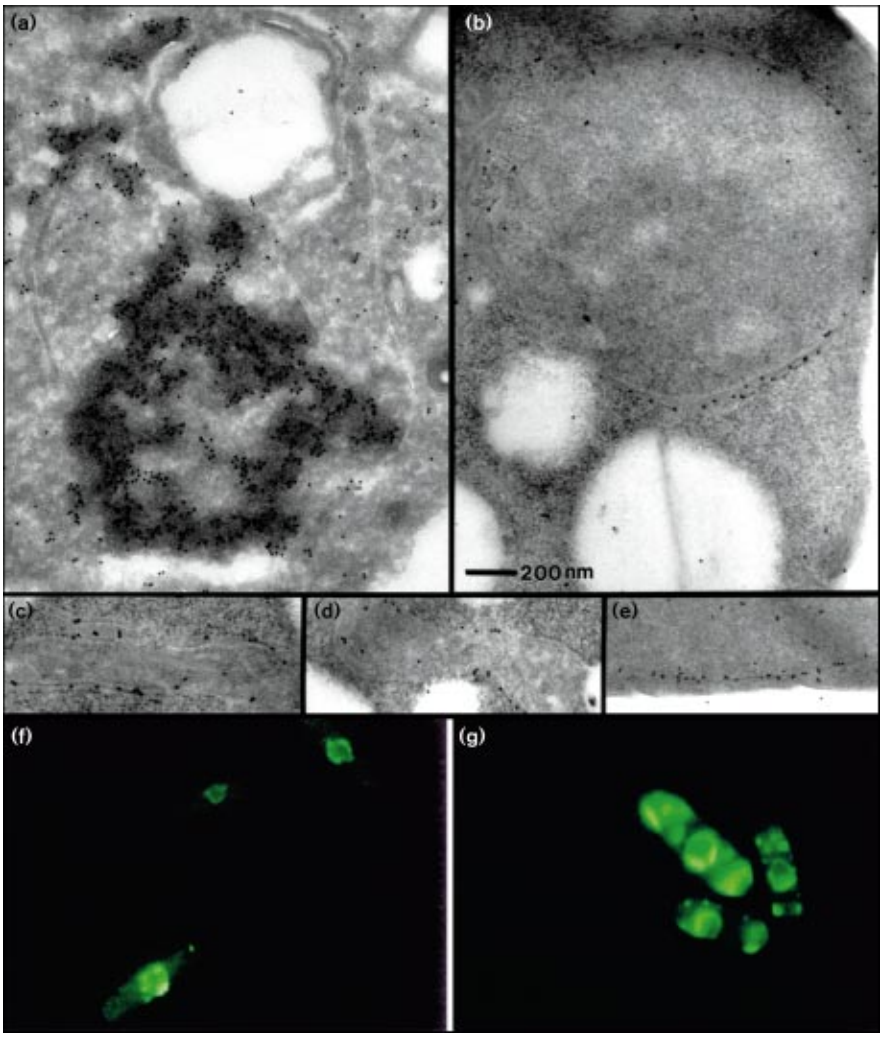
A cDNA encoding the full-length open reading frame of the pro-apoptotic splice variant of *ced-4* (*ced-4S*) fused to an amino-terminal haemagglutinin (HA) epitope tag (*HA.ced-4*) was placed under the control of the thiamine-repressible *nmt-1* promoter [13] and maintained as a multi-copy plasmid under uracil selection in *S. pombe*. Removal of thiamine from the culture medium resulted in induction of *HA.ced-4* expression after about five generations (approximately 12 hours at 32°C). Induction of *HA.ced-4* led to rapid slowing of culture growth, whereas control yeast cultures continued to proliferate rapidly (Fig. 1a). The viability of cells expressing *HA.ced-4* was assessed by plating them back onto agar supplemented with thiamine, which represses the *nmt-1* promoter-driven expression of *HA.ced-4* (Fig. 1b). By this criterion, the viability of cultures expressing *HA.ced-4* declined steeply to below 20% of that of control cells within 4 hours of induction and, by 24 hours after *HA.ced-4* induction, fewer than 1 in 10^4 cells were viable. To examine the phenotype of yeast expressing *HA.ced-4*, cells were stained with the DNA-binding fluorochrome 4-6-diamido-2-phenylindole (DAPI). Cells expressing *HA.ced-4* had greatly enlarged nuclei with an abnormally heterogeneous distribution of DNA (Fig. 1c,d). Electron microscopy confirmed that the chromatin in these cells was condensed into multiple foci (Fig. 1e–h). Ultrastructural localization of HA.CED-4 in these cells was determined by immunogold labelling and showed that HA.CED-4 protein was present on the condensed chromatin (Fig. 2a). Thus, expression of

Figure 1



Expression of *HA.ced-4* suppresses culture growth, reduces viability of *S. pombe* and induces chromatin condensation. (a) Equivalent cultures of *S. pombe* carrying *HA.ced-4* under the control of the *nmt* promoter were grown in liquid culture either in the presence (*HA.ced-4* expression repressed) or absence (*HA.ced-4* expression induced) of thiamine. At the time points indicated, samples of the cultures were removed and cell numbers counted with a Coulter counter. Cell numbers are shown plotted against time after thiamine manipulation. (b) Viability of *S. pombe* cells carrying *nmt-HA.ced-4* in liquid cultures either in the presence, or following withdrawal, of thiamine. Induction from the *nmt-1* promoter takes place approximately 12 h after thiamine withdrawal in a log-phase culture growing at 32°C. (c,d) *S. pombe* cells stained with DAPI. (c) Control (+ thiamine) cells with *HA.ced-4* expression repressed. (d) Cells expressing *HA.ced-4* 20 h after thiamine withdrawal, exhibiting enlarged nuclei and heterogeneous DAPI staining. (e–h) Electron micrographs of nuclei of *S. pombe* stained with osmium tetroxide. (e) Control (+ thiamine) cells with *HA.ced-4* expression repressed. (f–h) Cells expressing *HA.ced-4* 20 h after thiamine withdrawal and showing details of condensed chromatin.

Figure 2



Localization of HA.CED-4 and GFP.CED-4. (a–e) Ultrastructural localization of HA.CED-4 in *S. pombe* cells visualized by immunogold labelling using anti-HA monoclonal antibodies. Cells were isolated and processed for immuno-electron microscopy 18 h after thiamine withdrawal. (a) *S. pombe* expressing HA.CED-4 alone (paraformaldehyde-fixed cryosection with the anti-HA antibody 16B2 as first layer reagent): HA.CED-4 localizes to condensed chromatin. (b–e) *S. pombe* co-expressing HA.CED-4 and CED-9 (Lowicryl section with the anti-HA antibody 12CA5 as first layer reagent): HA.CED-4 localizes to nuclear membrane and perinuclear ER (b), mitochondrial membranes (c,d) and periplasmic ER (e). (f,g) Localization of GFP.CED-4 in *S. pombe*. (f) GFP.CED-4 expressed alone: GFP.CED-4 localizes to the nucleus. (g) GFP.CED-4 coexpressed with CED-9: GFP.CED-4 localizes to the cytoplasmic compartment.

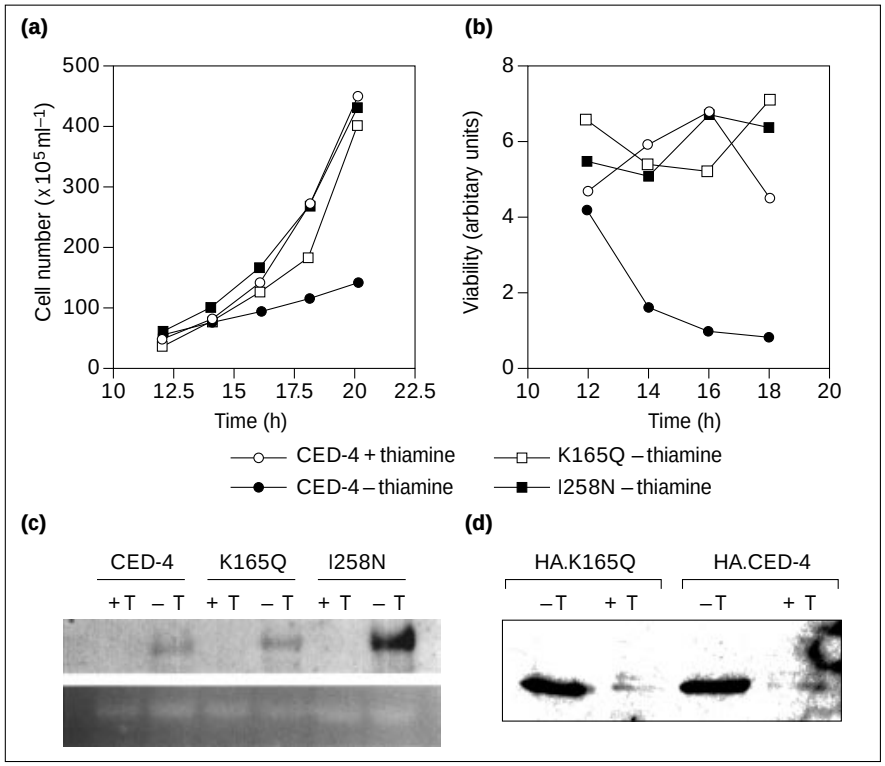
ced-4 in *S. pombe* induces lethal chromatin condensation: co-localization of CED-4 protein with condensed chromatin suggests that condensation of chromatin is a direct effect of CED-4 action.

Analysis of lethality of CED-4 mutants in *S. pombe*

The CED-4 protein has no reported homology with any known protein, nor does it contain any recognizable protein motifs: early reports of weak homology with the 'EF hands' present in some calcium-binding proteins have not been confirmed by any biochemical evidence. However, searching for motifs using the program PROMOT indicated that a region comprising residues 158–165 conformed to a potential nucleotide-binding P-loop consensus (A/GXXXXGKS/T, using the single letter amino-acid code) [14]. To investigate the functionality of this region, the putative critical lysine residue within this consensus was mutated to a glutamine residue (K165Q) by *in vitro* mutagenesis. Expression of this CED-4 mutant had a negligible effect on growth, viability or phenotype of *S. pombe* (Fig. 3), indicating that the putative P-loop is required for CED-4 function. Western blot analysis indicated that HA-tagged K165Q protein was present at similar or higher levels than the wild-type protein (Fig. 3d), establishing that the lack of phenotype resulting from K165Q expression was not due to instability of the mutant protein. We also used site-directed mutagenesis to reconstruct an established null mutant of CED-4 that had been identified in screens of *C. elegans*. Expression of this null I258N point mutant also had no effect on *S. pombe* cells (Fig. 3). Thus, the cytocidal activity of this CED-4 mutant is similar in both *C. elegans* and *S. pombe*.

Although chromatin condensation appears to be a ubiquitous feature of metazoan apoptosis, an effector specifically responsible for this phenotype has yet to be identified. Our data are consistent with the notion that CED-4 can condense chromatin by an intrinsic catalytic

Figure 3



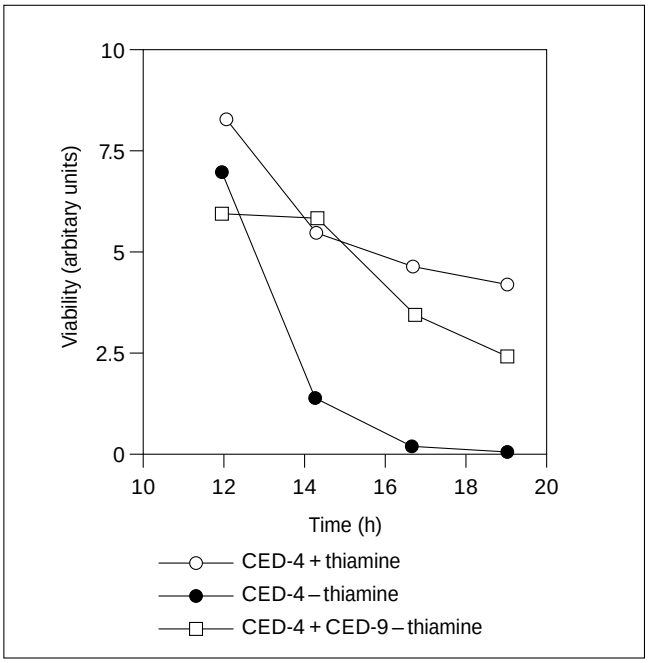
The *ced-4* mutants *ced-4K165Q* and *ced-4I258N* are not cytotoxic in *S. pombe.* (a) Growth of *S. pombe* expressing different CED-4 mutants in liquid culture following induction of expression. Cell numbers are plotted against time. (b) Relative viability of *S. pombe* expressing different CED-4 mutants in liquid culture at various time points following induction of expression. (c) Northern blotting analysis of *ced-4* expression in *S. pombe*.Total RNA from *ced-4*, K165Q and I258N *S. pombe* cells, either growing in the presence of thiamine (+ T) or 14 h after thiamine withdrawal (– T), was probed with a ^{32}P-labelled random-primed *ced-4* cDNA probe. Total RNA loaded and stained with ethidium bromide before blotting is shown underneath as a loading control. (d) Western blotting analysis of HA.CED-4 and HA.K165Q protein in *S. pombe*. Lysates of *S. pombe* cells either growing in the presence of thiamine (+ T) or 14 h after thiamine withdrawal (– T) were probed with 12CA5 anti-HA antibody.

activity that requires nucleotide binding to the putative P-loop of CED-4.

CED-4 induced lethality in *S. pombe* is blocked by CED-9

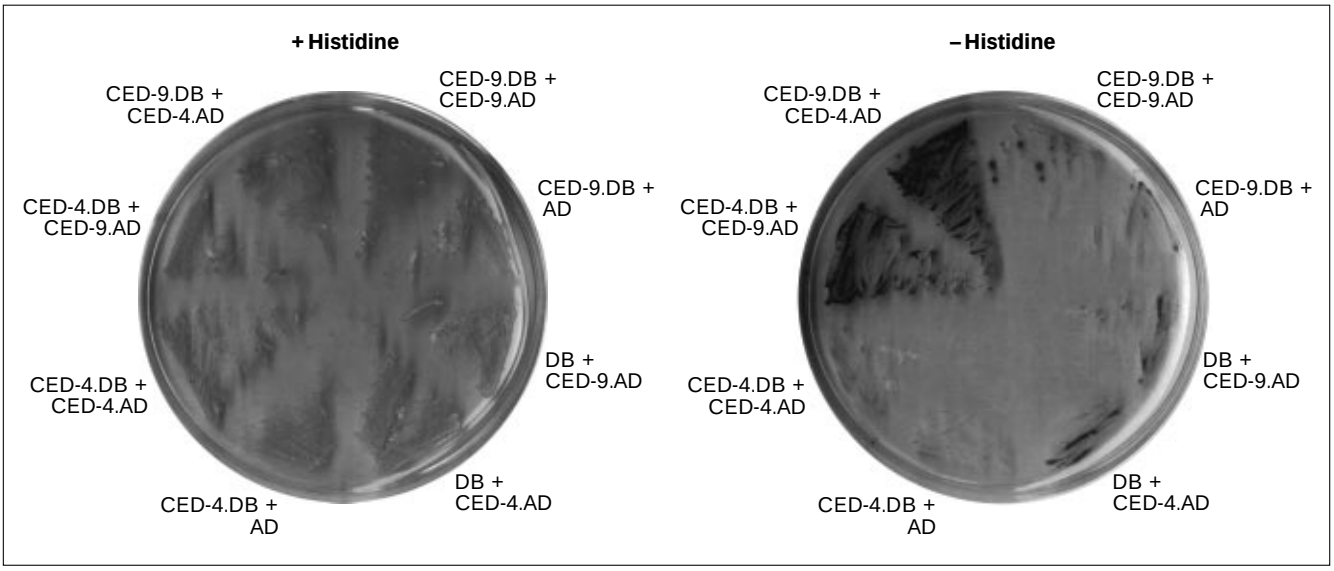
We next assessed the effects of known suppressors of apoptosis on the CED-4 lethal phenotype. We examined the abilities of *ced-9*, the gene encoding the baculovirus caspase inhibitor p35 [15,16], and the anti-apoptotic splice variant of *ced-4*, *ced-4L* [17], to inhibit HA.CED-4-induced cell death in *S. pombe* by co-expressing these genes with *HA.ced-4*. Neither *ced-4L* nor *p35* prevented CED-4-induced cell death or chromatin condensation (data not shown). In contrast, co-expression of *ced-9* significantly protected *S. pombe* cells from the lethal effects of HA.CED-4 (Fig. 4). Compared with cells expressing *HA.ced-4* alone, cells co-expressing *HA.ced-4* and *ced-9* exhibited increased growth rates (data not shown), long-term viability (Fig. 4) and a complete absence of chromatin condensation. Immunogold labelling of HA.CED-4 in *HA-ced-4*/*ced-9* co-expressors showed that, in this context, CED-4 no longer localizes to chromatin, but is instead localized to the endoplasmic reticulum (which is periplasmic and perinuclear in *S. pombe*) and outer mitochondrial membranes (Fig. 2b–e). CED-4 fused to green fluorescent protein (GFP) [18], GFP.CED-4, had a similarly altered localization when co-expressed with *ced-9* (Fig. 2f,g). Although the subcellular localization of CED-9 protein has not been well established, it shares

Figure 4



Co-expression of *ced-9* protects against *HA.ced-4*-induced lethality. Relative viability of *S. pombe* cells expressing either HA.CED-4 alone or both HA.CED-4 and CED-9 is shown at various time points following HA.CED-4 induction by thiamine withdrawal.

Figure 5



Interaction between CED-9 and CED-4 in the yeast two-hybrid assay. Colonies of *S. cerevisiae* strain HF7C were streaked out on medium containing histidine and lacking histidine. Yeast co-transformed with plasmids encoding fusions of CED-9 and the GAL4 activation domain (CED-9.AD) and CED-4 with the GAL4 DNA-binding domain (CED-4.DB), and those co-transformed with CED-4.AD and CED-9.DB were able to grow normally on medium without histidine, whereas those transformed with either CED-9.AD or CED-4.AD and the empty GAL4 DB vector, or CED-9.DB or CED-4.DB and the empty GAL4 AD vector did not grow, or grew very slowly on this medium. Interaction of the proteins fused to the activation and DNA binding domains of GAL4 allows transactivation of the *his* reporter gene, and hence growth on medium without histidine. Yeast co-transformed with CED-9.DB and CED-9.AD, or CED-4.DB and CED-4.AD, were also unable to grow on this medium, indicating that neither full-length CED-4, nor CED-9 lacking its transmembrane domain, self-associate in this context.

close structural features with its homologue Bcl-2, including a carboxy-terminal hydrophobic tail which ocalizes Bcl-2 to the endoplasmic reticulum and outer mitochondrial and nuclear membranes.

The re-localization of HA.CED-4 resulting from co-expression with CED-9 suggests that CED-9 may prevent HA.CED-4-induced death by directly binding and sequestering HA.CED-4. We therefore evaluated the ability of CED-9 and CED-4 to interact directly by a yeast two-hybrid analysis in which physical association between CED-4 and CED-9 is required for propagation of yeast on medium lacking histidine [19]. Expression of either CED-4 or CED-9 fused to either the DNA-binding domain (DB) or activation domain (AD) of GAL4 in the *Saccharomyces cerevisiae* strain HF7C did not permit growth in the absence of histidine. In contrast, co-expression of CED-4 and CED-9 fusions led to rapid growth (Fig. 5), indicating that the two proteins interact. Co-expression of the CED-4 and CED-9 fusions with heterologous partners (p53.DB and SV40 T Ag.AD) permitted only very slow growth, suggesting that the interaction was not due to non-specific 'stickiness' of CED-4 or CED-9 (data not shown). Neither co-expression of CED-4.DB and CED-4.AD, nor co-expression of CED-9.DB and CED-9.AD were able to support growth on medium lacking histidine, suggesting that, in this context at least, neither CED-4 nor CED-9 self-associate.

Conclusions

Our data support the notion that CED-4 (and its putative vertebrate homologues) has a direct role in driving a caspase-independent condensation of chromatin during apoptosis and indicate that this function can be reproduced in *S. pombe*. This notion is further supported by the fact that neither a known null point mutant of CED-4 nor CED-4 mutated in its putative P-loop induces chromatin condensation or loss of viability in yeast.

Vertebrate apoptosis, once triggered, involves a plethora of cellular processes that are presumably implemented by different effectors. Indeed, several lines of evidence suggest that some of these processes can occur independently: for example, membrane blebbing and cell vacuolization do not seem to require caspases [20,21]. The observation that two known metazoan death promoters, CED-4 and Bak/Bax [12], are both able to induce death of *S. pombe* but with very different phenotypes (chromatin condensation and vacuolization, respectively) may reflect the fact that *S. pombe* is a genuinely naive yet useful vehicle for the analysis of the different processes that together comprise metazoan PCD. Irrespective of this,

however, our demonstration of a direct interaction between the pro-apoptotic CED-4 and anti-apoptotic CED-9 proteins, resulting in the sequestration and relocalization of CED-4 away from the nucleus and into the intracellular membranes, reveals an unequivocal mechanism for the anti-apoptotic activity of CED-9. Further studies are required to establish the influence of the CED-9/CED-4 interaction on the activation of CED-3.

Materials and methods

Cloning and in vitro *mutagenesis*

The cDNAs encoding full-length CED-4S (*ced-4*), CED-4S amino-terminally tagged with the HA epitope (*HA.ced-4*) and a GFP–HA.CED-4 fusion were generated by PCR using *BioTaq* polymerase and the manufacturer's recommended PCR conditions. Sequence was verified by manual sequencing according to standard protocols. The *HA.ced-4* cDNA was cloned downstream of the *nmt-1* promoter in pREP4X which carries a uracil marker. The pALTER system (Promega) was used for *in vitro* mutagenesis according to the supplied protocol. The mutagenic oligonucleotides were GCCCAACGATTTGTTTCTTCTTG-AAC (I258N) and CAATTACTGATTGTCCGGATCCAG (K165Q). The *ced-9* and *ced-4L* cDNAs were kindly provided by Robert Horvitz and cloned under the control of the *nmt*-1 promoter in the vector pREP41, which carries a leucine marker. The mutated GFP cDNA was kindly provided by Jon Pines.

Yeast culture

The *S. pombe* strain 567, auxotrophic for uracil, adenine and leucine, was transformed by electroporation and grown in selective medium ± 100 ng ml^{-1} thiamine. To induce the *nmt-1* promoter, log-phase cultures were extensively washed in medium without thiamine, diluted to an OD_{595} of 0.0125 and grown at 32°C. At each time point, the culture density was determined using a Coulter counter. A 10 μl aliquot of a 1/100 dilution of the liquid culture was plated onto selective plates supplemented with thiamine, grown at 30°C for 4 days and the number of colonies on the plate counted. Viability was calculated as the number of colonies formed over the number of cells plated out, as indicated by the Coulter counter reading for that time point.

Northern blotting analysis

Total RNA was extracted from yeast cells with TRIzol (Life Technologies) after disruption of the cell walls with acid-washed glass beads. RNA (10 μg per lane) was fractionated on a 2% formaldehyde, 0.8% agarose gel and blotted onto HybondN$^+$ nylon membrane (Amersham). A ^{32}P-labelled *ced-4* cDNA probe was generated using the Multiprime DNA-labelling system (Amersham). Filters were hybridized at 55°C in CHURCH buffer (342 mM Na_2HPO_4, 158 mM NaH_2PO_4, 7% SDS, 1 mM EDTA) and washed at 65°C in 0.5× SSC, 0.1% SDS.

Western blotting analysis

Proteins were extracted from yeast cells with TRIzol (Life Technologies) after disruption of the cell walls with acid-washed glass beads. Extracts were separated by electrophoresis through a 10% SDS acrylamide gel and blotted onto ImmobilonP membrane. The blots were blocked and incubated with antibodies at room temperature in TBS containing 2% milk and 0.5% Tween 20. The first layer reagent was 12CA5 anti-HA antibody, second layer was HRP-conjugated rabbit anti-mouse (Amersham). Protein–antibody complexes were visualized by ECL (Amersham).

Electron microscopy and immunogold labelling

To produce sections for morphological analysis, yeast cells were prepared using conventional cryo-morphology methods with the addition of a 1% osmium tetroxide step after the initial aldehyde fixation. For immunolocalization analysis, cryosections were prepared and labelled using the three-stage method of Slot *et al.* [22]. Sections of Lowicryl HM20 were prepared by progressive lowering of temperature [23] and labelled as above. HA-tagged antigens were detected by overnight incubation at 4°C of sections with appropriately diluted anti-HA monoclonal antibodies 12CA5 and 16B2. Binding of anti-HA monoclonal antibodies was detected by incubation for 30 min at 25°C with a 1/200 dilution of rabbit anti-mouse IgG1 (Nordic) followed by incubation for 30 min at 25°C with Protein A conjugated to 10 nm gold (Utrecht University) at a dilution of 1/50.

Yeast two-hybrid analysis

In-frame fusions of CED-9 (truncated at Lys247) with the GAL4 DNA-binding domain (CED-9.DB) and the GAL4 activation domain (CED-9.AD) and CED-4 with the GAL4 DNA-binding domain (CED-4.DB) and the GAL4 activation domain (CED-4.AD) were generated by PCR, their sequence verified by manual sequencing according to standard protocols and the vectors transformed into the *S. cerevisiae* strain HF7C. HF7C is auxotrophic for histidine, but carries a *his* reporter gene that is transactivated by GAL4.

Note added in proof

Recent papers by Spector *et al.* (*Nature* 385:653–656), Chinnaiyan *et al.* (*Science* 275:1122–1126) and Wu *et al.* (*Science* 275:1126–1129) have demonstrated the existence and functional significance of the CED-4–CED-9 interaction. Chinnaiyan *et al.* have further shown that CED-4 is capable of interacting with CED-3, indicating a possible mechanism for CED-9 to influence the activity of CED-3.

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